

Author's response to reviews

Title: Thyroid function in Danish greenhouse workers

Authors:

Gunnar Toft (gutof@as.aaa.dk)
Allan Flyvbjerg (allan.flyvbjerg@dadlnet.dk)
Jens P Bonde (jpbond@as.aaa.dk)

Version: 2 Date: 9 October 2006

Author's response to reviews: see over

Dear Editors at Environmental Health, Professor Philippe Grandjean and David Ozonoff.

We would like to thank you and the reviewers for the excellent comments to our manuscript, which we believe have markedly improved the manuscript. Below follows detailed answers to each of the reviewers comments. We look forward to hear whether you can accept the manuscript based on the introduced changes.

The coauthors have read the manuscript in its present form and accepted the changes.

Yours sincerely,

Gunnar Toft

Answer to reviewer's reports (reviewers comments in italics and)

Reviewer: Larissa Takser

General

The research subject is topical and well defined. The paper addresses the risk of thyroid disruption in pesticides exposed greenhouse workers. However, statistical analysis and results presentation should be improved. Also, the interpretation of data is significantly limited by lack of true control group.

Thank you for your comments. Please see our answers to the specific issues below.

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

Background: The brief information about chemical structure, the existing groups of pesticides, and the mechanism of thyroid disruption by these chemicals should be introduced. What are the most potent thyroid disruptors among these substances?

Since the greenhouse workers are exposed to a mixture of numerous pesticides it is not possible to describe in detail the chemical structure and mechanism of thyroid disruption of all these compounds. Furthermore, the mechanisms have not been investigated for a number of these pesticides. Therefore the mechanisms of pesticides on the thyroid are described in general terms on page 3. However, in the 'Discussion' we describe in more details possible mechanism of action with reference to a new review. The following sentence has been removed to the 'Background' section page 3:

Pesticides may interfere with thyroid hormone homeostasis through many mechanisms of action, i.e. at the receptor level, in binding to transport proteins, in cellular uptake mechanisms or in modifying the metabolism of thyroid hormones (reviewed in [1]).

The demonstrated thyroid disrupting effects of the pesticides used by the greenhouses are described in Table 2. It is difficult to classify which are the most potent since all chemicals are not assessed in one standard test. However, the most concerning finding for human health is the association between the chlorpyrifos metabolite and thyroid hormones in a general population of American males. The following sentence is introduced on page 6:

Among these, the only demonstration of effects on thyroid hormones among humans was observed in association to the chlorpyrifos metabolite 3,5,6-trichloro-2-pyridinol (TCPY) [9], but the effects of most of the compounds are unknown.

Method: It is not clear if thyroid or other endocrine disease were included in the study. The information about these disorders in recruited workers should be given. The free version of the reference [7] is not always available. I would like to have more details about the recruitment: the response rate, the method of recruitment.

The section on recruitment on page 4 has been revised:

The participants were recruited by contacting greenhouse owners of 38 greenhouses with 3 or more employees in the autumn of 1993. Thirty-four of the greenhouse owners permitted a meeting for all male workers at their workplace, where we informed about the project. We invited all 199 male workers who fulfilled the inclusion criteria (age 18-45 years, normal puberty, no vasectomy, no known azoospermia, no malignant disease, and no intake of sulfasalazine, β -adrenergic blocking drugs or androgens) to sign-up for the study. A total of 122 men (61%) agreed to participate. None of the men included in the study had known thyroid disease or diabetes. The characteristics of the study population is described in Table 1.

The criteria of exposure, current and chronic, as well as variables used in statistical analysis should be more detailed. For example, "spraying load" should be defined in the method section before its use in tables.

The labels of the definitions of exposure measures have been made more specific to clarify the relation to exposure groups used in the tables.

1) *Spraying load*: Measured at the greenhouse level as the area sprayed in a 3 month period in the summer representing the main spraying period divided by the total greenhouse area. 2) *Spraying hours*: The average number hours actively spraying per year for each individual.

3) *Glove use*: The use of gloves or not during work with culture plants, characterized as always (n=20), sometimes (n=48) and never (n=53).

4) *Exposure years*: The number of years worked in a greenhouse.

5) *Job task*: The greenhouse workers were in addition classified according to job tasks and work practice in three groups taking into consideration all aspects of job function and exposure to evaluate the transfer of pesticides to individuals. This evaluation was performed by 2 agronomists and one occupational health physician. The groups can be characterized as follows: 1 - Low level exposure - working with administration, care and surveillance, pot machine, moving tables and newly hired (n=44); 2 – medium level exposure - working with packing, care and surveillance, spacing cultures, pricking and potting (n=45), and 3 – high level exposure - working mainly with nipping and cutting cuttings (n=13). In addition to job task estimated transfer of pesticides the amount of pesticides used in the greenhouses were used to estimate the individual exposure. A more detailed description of the classification can be found in [15].

Table 1: Why the population characteristics are compared between "spraying load" groups? If no true reference group is recruited, the characteristics of all recruited workers can be given in the Table with comparisons given in the text. What are the drugs consumed by this population? Do the characteristics vary between summer and autumn?

Table 1 has been reduced to one column for all greenhouse workers. Unfortunately we do not have specific information on the drugs used by this population, and we do only have one measure of these characteristics which (except for medicine use last month) are assumed to be rather stable within one year.

Table 2: The third column should be removed. We believe the information about known thyroid disrupting effects of the pesticides used in the greenhouses is important. However, we can accept to move the information to the text. We therefore delete the column and insert the following sentence in the text (page 5): **Thyroid disturbing effects have been demonstrated for the following insecticides: deltamethrin, endosulfan and chlorpyrifos [10-12] and the following fungicides: carbendazim, vinclozolin and thiram [13-15]**

The Table should provide the available information about individual exposures, not only greenhouse use.

The use of pesticides by name and number of applications are only available on the greenhouse level. The individual spraying activity is not necessarily a good measure of individual exposure, since a major part of the exposure comes from handling sprayed cultures – especially if handled without gloves. *How many pesticides are currently used by workers?* Two of the compounds in Table 2 (Vinclozolin and Thiram) has been prohibited for use since the collection of samples for this study. (this information is included on page 5) *Does this exposure vary between seasons?* The use of pesticides varies between seasons in the study period and presently.

Statistical analysis and Results: What are the dependent variables and the independent variables in the final models?

The last sentence of the statistics is changed to: **“For the exposure measures that could be determined on a continuous scale additional regression analysis for the association between the exposure measures (spraying load, spraying hours and exposure years) as independent variables and the thyroid hormones as dependent variables were performed.”**

Does the studied association change if adjusted for gloves use? Can the use of any protection be potential confounding factor (i.e. the highly exposed workers use the gloved more frequently than moderately exposed workers)?

Initially we did not adjust for glove use in the models. However the use of gloves was included in the evaluation of exposure based on job tasks. When adjusting the association of spraying load, spraying hours and exposure years to thyroid hormone concentration for glove use we found essentially the same results. Furthermore glove use did not qualify as a confounder by the criteria used. Therefore we prefer to present the results as they are.

Why the results are expressed as means and 95%CI? If regression models are used, the parameters of these models are more appropriate.

Only models with continuous measure of exposure could be presented as regression models (spraying load, spraying hours and exposure years). To keep the format simple we prefer to present all data from the exposure measures in categories in the tables and then additional results from the regression analysis are presented in the text, when relevant.

To avoid the confusion, the data should be presented separately for cross-sectional and longitudinal analyses. In the results section we describe cross sectional and longitudinal analysis separately, but to give an overview of the data we believe the present tables are sufficient. It would require several more tables with repeats of almost the same information to present the data separately.

For longitudinal analysis, I suggest to use the multivariate statistic. In the longitudinal analysis we compare data from the same individuals in the spring and the fall. Therefore it is irrelevant to control for age, BMI, and other lifestyle factors, which can be assumed to be constant within an individual

during that period. Therefore we used simple paired t-tests to compare the spring and the fall sample, and believe it is appropriate.

Discussion:

It is not always clear which results were significant. The statistical power of the study should be addressed.

The statistical significant results are marked in the tables with a letter and a footnote describes these, furthermore these findings are described in the text. We believe we have a sufficient sample size for most of the analysis performed. The sample size in the groups not simply base on dichotomization are now presented in the description of the exposure measures. In addition we include in the discussion the following two sentences: **“Finally, the sample size in some of the groupings is quite low. Especially the number of individuals classified as high level exposure based on job tasks (n=13) provide limited power. However, we believe the different approaches to exposure estimation would reveal effects of pesticides on thyroid hormones among greenhouse workers, if any. It should, however, be acknowledged that type and level of exposure might be much higher in other settings with less stringent exposure control than in Denmark”**

Page 11, third paragraph, second sentence: It is evident that an epidemiological study can not determine the exact mechanism of thyroid disruption. I suggest removing this sentence.

The sentence has been removed.

I agree with the authors about the need of an external control group. This is the major limitation of the study.

If this limitation can not be corrected, the authors can give the information about the laboratory values of thyroid hormones in population similar the study group measured by the same laboratory.

The reference range presented in supplementary table 1 is based on a sample from the general population. Unfortunately, we do not have access to the data at the individual level of these persons, and using patients as a reference would give a misleading figure since they are only referred to thyroid hormone analysis if altered thyroid function is suspected.

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

Background:

first paragraph, 7th line: The sentence “Except for the decrease...” is not clear.

The sentence has been revised to: **“Except for the decrease in FT4 in the latter study, these findings can be explained by an effect on thyroid function at the peripheral level causing a decreased level of circulating thyroid hormone and consequently an increase of TSH by negative feedback mechanisms.”**

Discussion:

first paragraph, first sentence: the level of FT4 was decreased, not elevated, from Table 4.

Thanks for noticing this mistake. It has been corrected.

Reviewer's report

Reviewer: John D Meeker

General

The manuscript describes a study that explored associations between exposure to pesticides among Danish greenhouse workers and thyroid hormone levels. Hormone levels were measured in serum at two points in time, and exposure was estimated using several various classifications from information gained by questionnaire. Two sets of statistical analysis were conducted; cross-sectional and longitudinal. The authors found evidence of an association between pesticide exposure and declined T4 in the cross-sectional analysis, which is consistent with limited existing animal and human data. The authors also reported a decline in TSH and an increase in T3 and T4 from spring to fall in the longitudinal analysis, but no patterns were evident with respect to pesticide exposure groups. The utility of a longitudinal analysis in the study is limited due to the inability to assign separate exposure estimates for the subjects in the spring and in the fall, which would allow for the use of mixed-effects models. Similarly, the analysis is limited by the inability to assume with confidence that the spring is an "unexposed" or baseline measure to compare with the fall measure, which follows months of exposure. However, at the end of the background section the authors appear to make this assumption to form their hypothesis for the longitudinal analysis. This means that since there is no true "unexposed" reference group, season is considered an additional sixth (broad) index of pesticide exposure in the study (where spring = low exposure; fall = high exposure) and it is hypothesized that the relationship between season and thyroid hormones will be further modified by the more refined exposure classifications.

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

1. Without the availability of two exposure estimates for each individual, the results comparing spring and fall thyroid hormones, though interesting, are difficult to interpret. One interesting analysis the authors may be able to follow-up on is to explore the associations between exposure and thyroid hormones by season when also separating subjects by those who were likely exposed to potential thyroid hormone-altering fungicides (listed in table 2) from those who were not. Since the authors state that many times fungicides are used more heavily in the winter and spring, it is possible that including the fungicide-exposed subjects in the original analysis may have resulted in null relationships in part due to misclassification.

Only 6 persons from who we had samples in the spring and in the fall were not exposed to fungicides. Therefore the sample size is too small to make any meaningful investigations on this group alone.

Methods:

2. More detail in the methods is needed:

2.1. Blood was collected from 122 subjects in the spring and only 85 in the fall. What is the primary explanation for the substantial loss of follow-up (30%)?

The collection of semen samples were done at the greenhouses in a mobile laboratory. The major reasons for drop out was change of working place, absence from the greenhouse because of having to attend gardening school or closure of the greenhouse. This information has been included in the methods section.

2.2. Exposure groups: In the first exposure index approach that is presented, during the initial read-through of the manuscript it is difficult to determine if this ratio of treated areas to total area is measured by greenhouse or if it is measured for individual workers doing the actual pesticide application (i.e. could be different for 2 workers from the same greenhouse). Later on in the paper it becomes evident that it is by greenhouse, but this could be made clearer in the methods when introduced. It may also be easier to follow if you refer to this as exposure grouping #1 throughout the

text in addition to saying higher or lower "spraying load", as this can be confused with the second exposure index approach (hours spraying).

The different exposure indexes have been defined more clearly (see comments to Larissa Takser). We believe this should make it possible to distinguish between exposure measurements throughout the text.

2.3. In the table spraying load has a unit of times sprayed over 3 months, but in the methods it says this value is a ratio... please specify.

The spraying load is measured at the greenhouse level as the area sprayed in a 3 month period in the summer representing the main spraying period divided by the total greenhouse area. This definition is now given when first introduced, and deleted from the table due to the corrections above.

2.4. How was proportion of greenhouse area treated (approach #1) gained? Was this answered by each worker? Or possibly answered by a greenhouse manager or supervisor? If answered by the worker, were all answers by workers in the same greenhouse equal? What did the distribution of this variable look like?

Information on treated area and compounds was provided by the greenhouse owner, foreman or applicator (**one person per greenhouse provided the information**). The parenthesis is included where this is described in the methods section to clarify.

2.5. Did the size of the greenhouses vary? If sizes varied substantially, the use of the proportion of the greenhouse treated instead of total area may introduce additional exposure measurement error.

The areas of the greenhouses varied from 5000 to 50.000 square meters. For that reason we included both the spraying load at the greenhouse level (proportion treated) and the hours sprayed reported at the individual level. We admit that variations in greenhouse size may introduce measurement error at the individual level, but we believe the exposure is more accurately determined by the proportion of the greenhouse sprayed than the area sprayed (which is directly dependent on size).

2.6. Exposure approach #5 was a bit unclear: did it use information from the preceding exposure approaches or was it based solely on job title and expert judgement?

The exposure assessment named 'job tasks' used a combination of all accessible information to evaluate individual exposure. We have included a few more details in the description.

2.7. Along those same lines, was any attempt made to combine exposure assignment methods (sub-stratification or multivariate models)—at least the ones that are not highly related to one another? For instance, identifying individuals that sprayed substantially that also did not wear gloves, etc. It seems this would be feasible to explore with the available sample size.

The exposure approach #5 is a combined measurement. We do not believe we have sufficient power to make further substratification.

2.8. Statistical Analysis: Confounding variables-- were bivariate analyses run between these variables (age, BMI, etc.) and the exposure and outcome variables? Were there any interesting relationships worth mentioning?.

The bivariate outcomes on potential confounding variables is not the main focus and has therefore not been included. For your information, we did not find any significant associations between age, bmi, smoking, alcohol, coffee or medicine use and thyroid hormone levels in the cross sectional analysis.

Results:

3. Page 7: the results with decreased T4 when using spraying load as continuous scale are difficult to interpret. What is the unit by which spraying load was estimated? (i.e. need a more detailed description of what the regression coefficient represents).

The unit of FT4 and a parenthesis explaining the meaning of one unit increase in spraying load (indicating per time the total greenhouse area is sprayed) is included.

Discussion:

4. Page 10, line 5: "...period without iodine supplementation of table salt in Denmark..." Can you (briefly) provide more details for context?

The following sentence is added:

"Due to the relative high incidence of thyroid diseases it was decided to start iodine supplementation of table salt in 1998 in Denmark."

Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)

1. Discussion, page 9: First sentence should say FT4 was reduced, not elevated.

It has been corrected (also noted by Larissa Takser).

2. Discussion, Page 9, 2nd sentence of 3rd paragraph: Indicate that this exposure pattern between summer and fall previously observed in reference #9 was among the same workers that were included in this study.

The sentence has been changed to : **"In this population it has previously been demonstrated that the level of pesticide exposure is highest in the summer and fall period [16]"**.

3. There are a number of grammatical and spelling errors throughout that need to be corrected.

Some grammatical and spelling errors have been corrected

Reviewer's report

Reviewer: Vincent F Garry

General Report

Manuscript Title: Thyroid function in Danish greenhouse workers

Authors: Toft G., et al

Overall, the authors have put forward a technically sound well referenced manuscript.

However, there are some concerns.

Page 4

Blood samples..., 06.00 to 10.00 (Morning)

"AM" included

"competitive immunometric methods" Please cite reference and give added detail.

A reference (Hubner et al 2002) has been given, and the following specification of the methods has been included:

"The TSH third generation assay is a sandwich immunoassay using paramagnetic microparticles as solid phase and direct chemiluminescence of acridinium ester for detection of the signal. The assays for T4, FT3 and T3 are competitive immunoassays with SPALT (solid-phase antigen luminescence technique) architecture using paramagnetic microparticles as solid phase and direct chemiluminescence of acridinium ester for detection of the signal."

Page 5

"comprehensive questionnaire" Did you validate the questionnaire ?

“The performance of the questionnaire was tested in repeated pilot surveys among greenhouse workers and the validity of the questions were evaluated by interviews in a subgroup of workers that had filled in the questionnaire.” This information has been included in the text.

“ethichal” ? Thanks for noting the spelling error it is of course “ethical”

“newly hired” How does this equate with low level exposure? The evaluation based on job task is an overall evaluation of pesticide exposure and accumulation in the individuals. Newly hired have low accumulation of persistent pesticides.

Page 6

Fungicides used in a higher level in spring? On what crops or plants? Roses, seed potatoes ?

We do not have information on which plants the pesticides are used. However all greenhouses produced ornamental plants. The information on ornamental plant production is included in the first sentence in the methods section.

Page 7 *“Cary, NC”* ? Thanks for noticing: the correct town is Cary, NC.

Page 8 *“4 persons the measurements were consistently out of the reference range”*

Provide these data. These data might be indicative of disease.

As noted below in the results section, the most common deviation was increased TT3 without alteration of TSH. Also, the deviation was below action level in all cases, and therefore we do not believe it is an indication of thyroid disease. We include a table of deviating thyroid hormones as a supplementary table to the text, and refer to that in the results section.

For example, what is the frequencies of subclinical hypothyroidism or clinical hypothyroidism in the age group and sex of the persons you studied.?

Based on the information in a large study of thyroid function in the Danish population, approximately 1% of younger men should have subclinical hypothyroidism, and less than 0.5% should have clinical hypothyroidism [19].

This sentence is included in the discussion.

What does “only slightly “ mean?

The actual values are given in the supplementary table.

Page 12 *“ Seasonal differences” other causes than pesticide exposure” Can you give a better explanation or hypothesis? For example, were agricultural/horticultural pesticides commonly used throughout the geographic region of concern and how were they applied?*

“Other causes” in the conclusion refers to the text, where it is described that seasonal differences in food intake, or other unknown factors causing the variation in thyroid hormones which have also been observed in previous studies. The seasonal difference data does not directly indicate pesticide caused differences (due to the lack of association to exposure groupings). The difference between greenhouses in pesticide use is not in our opinion a likely explanation of seasonal differences in thyroid hormones.

The high exposure group you provided is poorly defined. Do you have any measurements; analytical (ambient or other) in the greenhouse, duration of workers in the greenhouse per day and number of days per year in the green house? These can provide some semi-quantitative estimate

The exposure estimates in the “job task” classification was supported by measurement of pesticides on gloves from workers during 8 tasks from 3 greenhouses. This information is added to the materials section.

Page 15

Table 1 Provide the number of persons in the low and high spray load groups and define

Table 1 has been revised no to include low and high spraying load.

In the statistics section we include the following sentence:

“Exposures that could be measured on a continuous scale were dichotomized at the median value to make two exposure groups of equal size.”

For other exposure groups the number in different exposure groups is defined in the text (methods section).

Page 17

Table 3 Account for differences in the number of subjects spring vs fall.

See comment to John D Meeker.

Please use paired samples from the same subjects for spring- fall comparisons.

Paired samples from the same subjects were used in the spring-fall comparisons throughout. This has been specified in the statistics section.

Table 4 same comments

See above

Table 5 Use paired samples only

See above

In all tables exclude all subjects (N=4) who were outside reference range both in spring and fall.

Exclusion of subjects outside of the reference range in both spring and fall did not change the level of significance of the findings in the analyses of differences between exposure groups. This information is included in the results section. We prefer to keep all samples in the analysis with this remark.

Were subjects referred for medical follow-up whose hormone level results remained outside the reference range?

As mentioned above we have no reason to believe that slightly increased TT3 without alterations of TSH was related to thyroid diseases. Therefore we did not contact these subjects for medical follow up.

Finally there were numerous English grammatical and spelling errors

The manuscript has been corrected for grammatical and spelling errors.
